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Inverse and correlative relationships between TRIBBLES genes indicate non-redundant functions during normal and malignant haemopoiesis

Tribbles expression in normal and malignant haemopoiesis

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Highlights

1. Significant negative correlation between *TRIB1-TRIB2* expression in haemopoiesis

2. *TRIB1-TRIB3* expression positively correlate in haemopoiesis

3. *TRIB3* expression has implications for haemopoietic stem cell quiescence

4. *TRIB1* expression is highest in the myeloid lineage

5. *TRIB2* expression negatively correlates with *C/EBP* family members

Abstract

TRIBBLES pseudokinases (*TRIB1, TRIB2 and TRIB3*) are important regulators of normal and malignant haemopoiesis. The relative abundance of each *TRIBBLES* family member may be important for distinct oncogenic or tumour suppressor functions. We map the expression profiles of *TRIB1, TRIB2 and TRIB3* in human and murine haemopoietic stem, progenitor and mature cells and in human leukaemia datasets. Our data show that *TRIB1-TRIB2* have an inverse expression relationship in normal haemopoiesis whereas *TRIB1-TRIB3* have a positive correlation. We reveal that *TRIB3* expression is high in the dormant haemopoietic stem cell (HSC) population, implicating a novel role for TRIB3 in stem cell quiescence. These analyses support a non-redundant role for each *TRIBBLES* member during normal haemopoietic differentiation. We show that *TRIB1-TRIB2* display a significant negative correlation in myelodysplastic syndrome (MDS) and acute myeloid leukaemia (AML) subtypes, but not in acute lymphoid leukaemia (ALL). This inverse relationship is specific to certain subtypes of AML. A positive correlation exists in different leukaemia subtypes between *TRIB1-TRIB3*. The *TRIB1-TRIB2* and *TRIB1-TRIB3* correlations are consistent with a correlative relationship with *C/EBP* transcription factor family members. Our results have implications for the development of strategies to therapeutically target these genes in different types of leukaemia.

Introduction

TRIBBLES proteins are pseudokinases that bind and modulate the activity of several signalling molecules, including kinases, phosphatases, transcription factors and components of the ubiquitin proteasome system. From their first identification in mammalian cells, *TRIBBLES* have been recognised as inducible genes, modulated by a wide range of mitogens and stressors, and associated with downstream regulation of key signalling pathways, including those of AKT, ATF4, NF-kB and MAPKs(1-3). TRIBBLES pseudokinases are involved in different disease pathways and in many cancer types, including solid tumours, such as melanoma, liver and lung cancer (4,5). The generation of *Tribbles* deficient mice has revealed important aspects of the diverse roles of the TRIBBLES proteins(6-10). Mammalian TRIBBLES proteins regulate differentiation and survival of haemopoietic cells across different lineages and are frequently deregulated in acute leukaemia(11).

The evolution of three mammalian family members, *TRIB1*, *TRIB2* and *TRIB3*, was suggested to be the result of gene duplications, from the ancestor *TRIB2* gene, that led to the acquisition of specialized protein functions and non-redundant biological roles(5). It has been shown that the three pseudokinases have both shared and unique biological functions, during normal and malignant haemopoiesis. TRIBBLES pseudokinases act as molecular scaffolds for the assembly and regulation of signalling modules and also as templates for substrate-directed ubiquitination. The TRIB pseudokinase domain is fused to a C-terminal tail containing binding sites for MAPK Kinase 1 (MEK1) (and other MAPKK dual-specificity kinases) and for the ubiquitin E3 ligase Constitutive Photomorphogenic Protein 1 (COP1)(12-14). The TRIB signalling output is controlled through the binding and modulation of active kinases e.g. MEK/MAPK, and through destruction of TRIB 'substrates' that interact via the pseudokinase domain e.g. Cell Division Cycle 25 (CDC25), Acetyl CoA carboxylase (ACC), and CCAAT/enhancer-binding protein (C/EBP) transcription factors(14-18). Structural biologists have confirmed the mechanistic cellular model and demonstrated that TRIBs C-terminal tail motif binds competitively in cis to both its pseudokinase domain and partner ubiquitin E3 ligases, such as COP1(19-21).

Recently it has been shown that oncogenic functions of TRIBBLES also include upregulation of anti-apoptotic B-cell lymphoma 2 (BCL2) levels, stabilization of *Promyelocytic leukemia-Retinoic acid receptor* α (PML-RARα), and p53-dependent upregulation of c-MYC, driving drug resistance(22-24). In contrast, tumour suppressor activity for TRIBBLES has been identified in AML and ALL, linked with defective p38 MAPK signalling(7,25,26). Thus, TRIBBLES pseudokinases appear to exert both oncogenic and tumour suppressive roles in different types of leukaemias. We do not yet know how these opposing roles for TRIB1, TRIB2 and TRIB3 are regulated, or when they manifest.

In this study, we interrogate publicly available expression data for TRIB1, TRIB2 and TRIB3 in human and murine normal haemopoietic cells, and leukaemia datasets using the BloodSpot platform (**Figure 1 and Supplemental Table S1**). We reveal specific inverse and correlative relationships between *TRIBBLES* family members in normal and malignant haemopoiesis. We correlate TRIB1 and TRIB3 positively, whereas TRIB2 correlates negatively, with C/EBP family members. Known (e.g. E2F(27)) and putative transcription factors(5) that control TRIBBLES expression distinguish the TRIB members in normal and malignant cells. Our analyses support that individual TRIBBLES have non-redundant lineage-specific functions during haemopoiesis. We provide evidence of a significant inverse relationship between TRIB1 and TRIB2 in the specification of different blood lineages and in AML inferred by their expression. We also identify high expression of TRIB3 in the highly self-renewing, dormant HSC population. Together our analyses show that TRIBBLES expression is differentially modulated in leukaemia and the direction (high or low expression) and the extent of expression variation is dependent on the oncogenic background of the malignant cells.

Results

Inverse relationship between TRIBBLES physiological expression levels in the haemopoietic system

To provide a comprehensive insight into *TRIBBLES* expression patterns in normal haemopoiesis, we interrogated the publicly available datasets of gene expression, using the bioinformatic platform BloodSpot, which combines expression data from curated microarray or RNA-Seq analyses of normal and malignant haemopoietic cells, at various developmental stages(28) (**Figure 1**). Firstly, we looked at curated human microarray data of the main blood populations, including haemopoietic stem cells (HSCs), early haemopoietic progenitor cells (HPCs), common myeloid progenitor cells (CMPs), granulocyte macrophage progenitors (GMPs), megakaryocyte-erythroid progenitors (MEPs), and differentiated myeloid and lymphoid cells (monocytes, polymorphonuclear cells (PMN) from PB, CD4+ and CD8+ T-cells, and natural killer (NK) cells) **(Figure 2, identified and isolated as described in Supplemental Table 2)**. The gene expression data provided by BloodSpot was combined from independent datasets (GSE17054(29), GSE19599(30), GSE11864(31) and E-MEXP-1242(32)) and was normalised and batch corrected, as described in (33). We found that both *TRIB1* and *TRIB2* had up to 6 Log2 expression units (eu) variation between their highest and lowest expressing cell types. Interestingly, they followed an inverse expression pattern. *TRIB1* was high in the myeloid lineage, with the highest expression value in differentiated CD14+ monocytes (9.5 eu), and expressed at low levels in the lymphoid cells, MEPs, and with lowest expression in the NK cells (3.7 eu). Conversely, *TRIB2* levels were high in the lymphoid populations, including B-, T- and NK cells (all ~10 eu), MEPs (10.5 eu), and low in differentiated myeloid cells (PMN and monocytes) (~4-5 eu). By contrast, *TRIB3* expression ranged within less than 2 eu, between 5.7 and 7 eu, with the highest expression values in the HSCs, CMPs, and GMPs and least expression in the T-cell compartment. Overall these data suggest that physiological *TRIB1* expression is important for differentiated myeloid cells, *TRIB2* for lymphoid and erythroid lineages, and *TRIB3* has a potential function in the more primitive stem and myeloid progenitor populations.

To correlate human expression data with the murine haemopoietic system, we used BloodSpot and retrieved gene expression data combined and batch corrected from two murine datasets of normal haemopoietic cells (GSE14833(34) and GSE6506(35)). The samples in this analysis included stem cells, progenitors, committed and differentiated populations of the myeloid, lymphoid and erythroid compartments, identified and isolated as described in **Supplemental Table 3.** *Trib1* expression during murine haemopoiesis followed a pattern similar to that observed in the human cells **(Figure 3A, and Supplemental Figure S1).** *Trib1* was low in the stem cells, lineage progenitors, erythroid, NK and T-cell lineages, and in differentiated B-cells, but highly expressed in the myeloid differentiated monocytes and granulocytes. Similar to expression data from human cells, murine *Trib2* expression levels were high in the T-, B- and erythroid cells lineages, and low in differentiated myeloid cells (**Figure 3B, and Supplemental Figure S1).** During T cell development, *Trib2* expression increased ~2 eu from the CLPs (4.2 eu) to the Early T-cell progenitor cells (ETP) (6.5 eu), peaked in differentiated single positive CD4+ and CD8+ T-cells (10.2 and 8.9 eu), and was slightly lower in their activated counterparts (populations activated with the lymphocyte mitogen Concanavalin A). During B-cell development, *Trib2* expression was low at early stages (Pro-B cells 4.4 eu), high in Pre-B and IgM+ B-cells (~10 eu), and reached ~8.5 eu in differentiated B-cells. The highest *Trib2* expression values were detected in the MkE precursor stage (11.2 eu) and were progressively lower in the derived populations of the erythroid lineage. Moreover, Trib2 was low in MkP cells (4.7 eu). The only cells that showed contradictory *Trib2* levels between human and murine data was in NK cells, where murine NK cells had low *Trib2* expression in contrast to high levels observed in human cells. Murine *Trib3* expression was high in the LT-HSCs, as observed in human cells, and in IgM+ B-cells, and low in the other compartments, including differentiated myeloid, erythroid, and lymphoid cells (**Figure 3C, and Supplemental Figure S1**). Higher *Trib3* expression was observed in activated T-cells (cells not present in the human datasets), compared to their naïve counterparts and other cell populations. To investigate further the expression of the three *Tribbles* genes in the stem cell compartment, we interrogated a dataset of murine active and dormant LT-HSCs(36). Interestingly, analysis with the GEO2R online platform revealed that only *Trib3* was included in the top 250 differentially expressed genes, with higher expression in the

dormant LT-HSCs, compared to the active counterpart (**Figure 3D**). These expression data may indicate a role for *Trib3* in regulating HSCs, and specifically HSC dormancy.

To more comprehensively address Tribbles expression in immune cells, murine *Tribbles* expression was evaluated in the microarray datasets of the ImmGen project(37-40), using BloodSpot (**Figure 4**). ImmGen represent a vast collection of expression data of over 250 FACS sorted haemopoietic populations from both myeloid and lymphoid lineages, innate and adaptive immune systems. For the purpose of our analysis, we focused on some key populations, among those available in BloodSpot, including those listed in **Supplemental Table 4**. In line with the previous results in both murine and human samples (**Figure 2 and 3**), *Trib1* had the highest expression in differentiated myeloid cells (monocytes 8.9 and granulocytes 9.5 eu). Again, *Trib2* showed the opposite lineage specific expression, with highest expression in the T- and B-cells lineages (ranging from 9.3 to 10.2 eu), and low in the myeloid compartment. Both Trib1 and Trib2 displayed high expression variability across different cell types (between 6.1 and 10.2 eu for *Trib2*, and between 6.3 and 9.5 eu for *Trib1*). *Trib3* expression range was again relatively narrow (from 6.6 to 7.2 eu), with high expression in the stem cell compartment. Once more we see an inverse relationship between *Trib1* and *Trib2* in the mature B cells, with high *Trib2* expression specifically in GC B cells (9.2 eu). Interestingly, the expression of all three mammalian *Tribbles* in CD4+CD25+FOXP3+ T-regulatory cells was higher than immature double-positive T-cells (**Figure 4**).

Furthermore, we looked at *Tribbles* mRNA expression in murine haemopoietic cells as measured by RNA-Seq (GSE60101(41)) and processed in the BloodSpot platform (**Supplemental Figure S2, Supplemental Table 5**). RNA-Seq data has several advantages over the microarray technology, including a higher signal-to-noise ratio and the possibility to detect a broader range of expression levels. *Trib1* was highly expressed in differentiated granulocytes (7 eu), moderately expressed in the stem and lymphoid compartments, and expressed at low levels in the erythroid lineage, similar to human and murine microarray data. *Trib2* was highly expressed in the T cells, with the highest expression values in CD4+ T-cells (6.9 eu), aswell as B cells, and MEPs, and progressively decreasing from

CMPs to GMPs to the lowest level in differentiated myeloid cells (<1 eu) all in line with the murine microarray results. An interesting contrast to the microarray data is the relatively high expression of *Trib2* in the murine HSC compartment in the RNA-seq data (**Supplemental Figure S2**). This has been confirmed, with qRT-PCR data showing higher *Trib2* expression in HSCs compared to GMPs(22). *Trib3* expression was overall lower in haemopoietic cells compared to *Trib1* and *Trib2*, with the highest expression values found in the stem cells, matching the microarray data.

Significant negative correlation between *TRIB1* and *TRIB2* in human AML subtypes

We next interrogated the relationship of Tribbles expression across leukaemias and in subtypes of human AML. Firstly, we examined the expression of the *TRIB1*, *TRIB2* and *TRIB3* genes, individually, across several leukaemia types collected and analysed from 5 independent studies (including data from GSE14468(42), GSE61804(43), GSE15434(44), the Leukemia MILE study (GSE13159(45)) and The Cancer Genome Atlas(46), TCGA, database, and collectively referred to as BloodPool(28), **Figure 1**). *TRIBBLES* expression patterns were analysed alongside normal haemopoietic populations at various differentiation stages (GSE42519(47)) (**Figure 5, Supplemental Figure S3 and Supplemental Table S6**). Visualization of the three genes' expression patterns, in the hierarchical plot format, allows us to compare the expression of each AML subtype with respect to several different normal haemopoietic lineages. *TRIB1* expression is high across most AML groups, with respect to normal HSCs and myeloid progenitor cells, but the expression rarely reaches the levels observed in normal differentiated myeloid cells which we have shown are the cells that express the highest levels of *TRIB1*. Similarly, in most AML cases *TRIB2* expression does not reach levels comparable to normal MEP cells which we showed express high levels of *TRIB2*. AML subtypes including deletions in chromosomes 5, del(5q), or 7, del(7q)/7q-, display higher expression than in normal HSCs or GMP, whereas in other AML groups *TRIB2* expression is lower than in normal GMP, e.g. AML subtypes with translocations of chromosomes 9 or 11. Finally, *TRIB3* expression range is again much narrower than *TRIB1* and *TRIB2*, and most AML subtypes have lower median *TRIB3* expression than normal HSC, CMP and GMP cells. Therefore, the comparison to control normal populations is

important for considering whether TRIB levels are high or low in different AML subtypes.

We calculated the Pearson r correlation coefficient, as a measure of gene correlation between TRIB1, TRIB2 and TRIB3 given their divergent expression in AML as well as in normal haemopoiesis. Firstly, in normal cells (all normal populations pooled), *TRIB2* is inversely correlated to both *TRIB1* and *TRIB3*, and *TRIB1-TRIB3* are positively correlated within each other (**Figure 6Ai**). Indeed, the Log2 expression values of *TRIB1* and *TRIB3* are comparable in HSCs (Figure 2, 3 and 5), which may indicate a positive regulatory relationship. Functionally there is a strong link between TRIBs and C/EBP family of transcription factors(48,49). Correlating the expression of *TRIB1*, *TRIB2* and *TRIB3* with *C/EBPA*, *C/EBPB*, *C/EBPD* and *C/EBPE* in normal cells, there is a positive correlation between *TRIB1* and *TRIB3* with all *C/EBP* members, and a negative correlation between *TRIB2* and all *C/EBP* members (Figure 6Bi and Supplemental Figure S6). Moreover, in all the samples in the BloodPool and in myelodysplastic syndromes (MDS), significant negative correlations for *TRIB1-TRIB2* and *TRIB2-TRIB3*, and positive correlations for *TRIB1-TRIB3* were found (**Figure 6Aii-iii**), and the correlations between *TRIBs* and *C/EBP* members are similar to those observed in normal cells (Figure 6Bii-iii). In acute lymphoid leukaemia (ALL), however, all three *TRIBBLES* were positively correlated with each other (**Figure 6Aiv**), and with *C/EBP* members (Figure 6Biv), suggesting that an inverse relationship between *TRIB1* and *TRIB2* exists only in myeloid (MDS/AML) leukaemias. Therefore, we analysed the Log2 expression data of the TRIBBLES family in AML subtypes. We observed an inverse correlation of gene expression between *TRIB1* and *TRIB2* in several AML subtypes including normal karyotype (NK) and complex karyotype AML, samples with common chromosomal translocations, such as inv(16) CBFB/MYH11, t(15;17) PML/RARα, 11q23/MLL, t(8;21) AML1/ETO AML, and other AML with chromosomal aberrations, such as -5/7q, -9q and Trisomy13 (although samples numbers were low in -9q and Trisomy13) **(Supplemental Figure S4A-I)**. Conversely, a positive correlation was found between *TRIB1* and *TRIB3* expression across all samples in the BloodPool dataset, and in the NK, inv(16) CBFB/MYH11, t(15;17) PML/RARα AML, and in other aberrant cytogenetics groups such as Trisomy 13 and -5/7q subtypes. *TRIB2* and *TRIB3* were inversely correlated only in AML samples with NK and -9q

(Supplemental Figure S4). Together, these data suggest that TRIB1 and TRIB2 are inversely regulated in myeloid cells and in AML and suggests that they may have non-redundant, or even mutually exclusive roles in such conditions. Additionally, the positive correlation between *TRIB1* and *TRIB3* in several AML subtypes suggests a cooperative relationship. We hypothesize that upstream regulators in the different AML subtypes influence *TRIB* expression. Previously we published the putative transcription factors that bind to the 5'UTR of each *TRIB* gene(5), some proven experimentally(27,50). We show here that the expression of these transcription factors clearly correlates differently with each *TRIB* member. In normal cells, the bloodpool and MDS samples, but not the ALL samples, we see similar correlation profiles for *TRIB1* and *TRIB3*, whereas the transcription factor correlation profile with *TRIB2* is distinct (Figure 6C). Therefore, the putative upstream regulators of the *TRIB* genes cluster *TRIB2* distinct from *TRIB1* and *TRIB3*, which may provide some explanation for the inverse and positive relationship between *TRIB1-TRIB2* and *TRIB1-TRIB3* respectively.

To gain more insight into the changes in expression in AML cells compared to normal cell expression levels, we assessed the expression patterns in the BloodPool vs Normal dataset, where the data from the BloodPool AML samples is provided after a further normalization level versus normal myeloid populations. Briefly, the expression from each individual AML sample in the BloodPool was batch corrected and normalised to that of the 5 nearest normal cells, according to Rapin's cancer vs normal (CvN) method, which is based on a 2-step principal component analysis (PCA) strategy, and gene expression fold changes were computed(28,47). Using this approach, we show that the median Log2 fold change variations (hereafter referred to as FC) expression of *TRIB1* is positive but actually only over a small range in AML samples compared to normal myeloid cells (between 2.7 and -0.7 FC) (**Figure 7 and supplemental Figure S5**). Of note however, t(15;17) PML/RARα AML has a median *TRIB1* expression FC that is smaller to that of all major AML subgroups (e.g. inv(16) CBFB/MYH11, t(8;21) AML1/ETO, 11q23/MLL rearrangements, t(8;16) MOZ/CBP AML). The analyses of the normalised *TRIB2* expression in several AML subtypes showed both positive and negative median FC values ranging from 3.2 and -2.8 (**Figure 7 and supplemental Figure S5**). Interestingly, *TRIB1* and *TRIB2* FC expression followed

opposite trends in several leukaemia subtypes. Namely, AML samples with chromosome 9 translocations (t(6;9) DEK/NUP214, t(9;11) MLL/MLLT3 and t(9;22) BCR/ABL1 AML) exhibited the highest median *TRIB1* expression FC amongst all the AML subtypes analysed, while they had a negative *TRIB2* FC. Moreover, *TRIB1*/*TRIB2* FC variations had an inverse direction (upregulated or downregulated, compared to normal myeloid cells) in MDS samples and in NK, t(8;21) AML1/ETO, t(11q23)/MLL rearrangements, t(8;16) MOZ/CBP, t(1;3) RPN1/PRDM16, del(9q) AML subtypes and samples with other AML translocations. In other aneuploidy cases, such as del(5q) AML, in complex karytotype AML and in ALL, both pseudokinases have increased expression, compared to normal myeloid cells (**Figure 7 and supplemental Figure S5**). Similar to our previous data, *TRIB3* gene expression had a limited range of FC variation (-1.2 to 1.2 FC), with respect to normal myeloid cells. Of note and in contrast to *TRIB1* and *TRIB2*, *TRIB3* has higher FC expression in AML patients with t(15,17) PML/RARα than in the majority of other AML groups (**Figure 7 and supplemental Figure S5**), which is a subtype of AML where this positive FC analysis has proved to be functionally significant(23,51). To further support the inverse correlation between TRIB1 and TRIB2, we determined the Pearson r correlation coefficient of *TRIBBLES* FC gene expression in the AML subtypes vs Normal dataset (**Figure 8**). Analyses of individual AML subtypes showed a significant negative correlation of *TRIB1-TRIB2* FC in NK **(Figure 8A),** inv(16) CBFB/MYH11 **(Figure 8B)**, t(15;17) PML/RARα **(Figure 8C)**, 11q23/MLL **(Figure 8D)**, -5/7(q) **(Figure 8E)**, but not in t(8;21) AML1/ETO AML samples **(Figure 8F)**. Moreover, significant positive correlations were found between *TRIB1-TRIB3* in NK AML subtypes **(Figure 8A)** and inv(16) CBFB/MYH11 **(Figure 8B)**, and a positive *TRIB2-TRIB3* correlation in patients with 11q23/MLL rearrangements **(Figure 8D)**. There is a disrupted profile of *C/EBP* expression in terms of correlation with *TRIBBLES* across the AML subtypes: it is more variable between the *C/EBP* family members in AMLs compared to the profile in normal cells, with the -5/7(q) subtype corresponding most closely to the normal profile (Supplemental Figure S7).

Discussion

Overall, the evaluation of *TRIBBLES* expression, in both human and murine gene expression datasets, highlighted distinct lineage-specificities of the three pseudokinases during haemopoiesis. *TRIB1* expression is higher in myeloid cells, compared to other haemopoietic compartments. *TRIB2* gene expression is upregulated during thymocyte development, in B-cells, and progressively downregulated from MEPs during erythropoiesis. Trib3 gene expression whilst appearing to be over a small expression range may be important for HSC dormancy. Our data suggest an inverse relationship between *TRIB1* and *TRIB2* in the specification of different blood lineages inferred by their expression, and suggesting non-redundant lineage-specific functions during haemopoiesis. Our data also clearly link *CEBP* family members with the inverse relationship between *TRIB1* and *TRIB2*. Additional transcription factors that bind to the different *TRIB* promoters may also contribute to the *TRIB* expression profile and their non-redundant functions. Without more data/samples of these populations we cannot reliably calculate correlation coefficients. These analyses are at steady state, and variations in expression levels may be altered in response to external stimulations, as in the case of lymphocyte activation.

Our analyses using AML data suggest that multiple cytogenetic aberrations can lead to *TRIBBLES* deregulated expression compared to their normal counterparts, particularly *TRIB1* and *TRIB2*. The inverse relationship between *TRIB1* and *TRIB2* is evident across different AML subtypes suggesting they are likely regulated through different oncogenic pathways. Indeed, this could be explained by transcription factor regulation of their expression, as putative factors appeared to cluster *TRIB1-TRIB3* distinct from *TRIB2*. There is an interesting clustering of *PPARa* transcription factor with a positive correlation with *TRIB2* and a negative correlation with *TRIB1* and *TRIB3* in normal cells. Overexpression data has shown overlapping roles for *TRIB1* and *TRIB2* in AML(12,52,53). Therefore, the non-redundancy of TRIB1 and TRIB2 in normal and malignant haemopoiesis is a necessary future line of functional investigation. Another possible explanation and not entirely mutually exclusive is that TRIB family members may be involved in regulating each other. Indeed a screen for protein interaction networks revealed the binding of TRIB2 to a previously validated E3 ligase target, COP1 linking to TRIB degradation function, and interestingly, to TRIB1(54). These

results suggest the formation of multiprotein complexes and potential reciprocal regulation between TRIBBLES members.

The generation of several strains of *Trib1*, *Trib2* and *Trib3* single KO mice (all able to reach adulthood) has been instrumental for studying the role of TRIBBLES in haemopoiesis(7,8,10,55-58). TRIB1-deficient mice exhibit abnormal differentiation of myeloid macrophage cells, with an increase in neutrophils and a decrease in macrophages observed in the BM and spleens of TRIB1 KO mice(8,56). In addition, *TRIB1*, *TRIB2* and *TRIB3* expression levels were shown to be dynamically controlled in response of inflammatory stimuli(59). The high expression of *TRIB2* in the lymphoid and erythroid lineages match the current literature demonstrating a role for TRIB2 in lymphoid and erythroid development: TRIB2 deficient mice lack proper T cell differentiation(6), and TRIB2 is important for early stages of erythrocyte development(10). Matching these data, it was previously shown that TRIB2 is the downstream target of the master regulators of megakaryocytic and erythroid differentiation, FOG and GATA1, and shown to be downregulated in differentiating erythroblast cells(60). *TRIB1* expression was absent in mature T-, B- and erythroid lineage cells, except for activated T-cell populations and in the CD4+FOXP3+CD25+ T-regulatory cells, which is in line with previously published data(61). The high similarity shared by three pseudokinases led to the hypothesis that compensatory mechanisms may be in place (at least in some cell types) in these murine models and mask some of the "deficient" phenotypes. The development of double and triple KO mice would therefore clarify the redundancy/non-redundancy in different cell types. Given the specific lineage distribution map of *TRIB1*, *TRIB2* and *TRIB3* defined by this study, it would be interesting to investigate whether deletion of one or two *Tribbles* would determine a redistribution of the other member(s) across the haemopoietic system.

Despite the small range and variability in TRIB3 expression in normal and leukaemic cells, it is interesting that *TRIB3* expression was higher in dormant HSCs which have the highest self-renewal potential compared to the active compartment(36). TRIB3 was identified as a negative regulator of the differentiation process of the haemangioblast (an early mesoderm precursor committed for haemopoiesis) into the haemopoietic progenitor cells (HPCs) (62).

Drosophila *tribbles* labels quiescent but not proliferating neural stem cells by promoting $Cdc25^{string}$ protein degradation during late embryogeneisis, and blocking activation of Akt in postembryonic neural stem cells(63), both mechanisms that have been linked with mammalian TRIBs(5,15). The role of TRIB3 in the adult HSC compartment has not yet been addressed and could represent a promising new field of investigation.

Our expression data suggest that the dosage of individual *TRIBBLES* genes is differentially regulated in specific AML subtypes. The reason that different AML subtypes have different expression of TRIB family members may be explained by differences in the upstream regulators of each TRIB. TRIB1-3 promoter regions contain very different putative transcription factor binding sites(5). Indeed, the *TRIB3* promoter has comparably less known transcription factor binding sites compared to *TRIB1* and *TRIB2* which may explain why *TRIB3* has the tightest dynamic range of expression. It could be speculated that these changes in expression, compared to the normal context, and the ratio between the three TRIBBBLEs influence the pathophysiology of the resultant AML disease. When we compared the expression pattern of the *TRIBBLES* across the leukaemia samples studied, and evaluated the presence of gene correlations between the expression (and the expression FC) of each member we uncovered a clear negative correlation between *TRIB1* and *TRIB2* and a positive correlation between *TRIB1* and *TRIB3* expression in the pool of AML, ALL and MDS samples studied (BloodPool dataset). Interestingly, chromosome 9 translocations have the highest fold change variations in TRIB1 which warrants further investigation. Chromosome 9 translocation include the genes *ABL*, *NUP*, and *AF9*, which link to deregulated *HOX* expression and TRIB proteins have been shown to cooperate with *HOX* expression(64,65). Moreover, we found that *TRIB1* and *TRIB2* expression are inversely correlated across several AML subtypes, suggesting that the two pseudokinases maintain an inverse regulation even in the cytogenetic groups where the expression of both genes was considered high (e.g.inv(16)) compared to other leukaemia subtypes. By analysing the correlations among *TRIBBLES* expression FC, from the BloodPool vs Normal dataset, we consistently found a negative correlation of the normalised expression of *TRIB1* and *TRIB2*, in the pooled samples and amongst several AML subtypes. Given that the expression values of each sample in this analysis were normalised against the

nearest normal population (FC values), these results suggest that the correlation between *TRIB1* and *TRIB2* is not just a consequence of the differential expression of the two genes in the normal haemopoietic populations, mirrored in their malignant counterparts. Instead, these data clearly indicate that there is an opposite trend in the regulation of the two genes in malignant condition with respect to normal haemopoietic cells.

Methods

The BloodSpot database was used to retrieve gene expression data from curated human and murine microarray and RNA-Sequencing (RNA-Seq) datasets(28)(Bagger et al. 2016) as summarised in **Figure 1**. Human and murine Trib1, Trib2 and Trib3 Log2 expression values were obtained from the BloodSpot database using the probes listed in **Supplemental Table S1**. These represent the probes with higher intensity in each analysis. Similarly, Log2 expression data for the relevant transcription factors were obtained using the probes listed in **Supplemental Table S2**.

The expression values or the FC expression values exported from BloodSpot were then graphed using GraphPad Prism software. To calculate statistical significance, each population was compared to the values from all other populations in the chosen analysis, except the one studied e.g. in Figure 2 CD14+ monocytes have significantly different *TRIB1* expression compared to the expression from all the other populations (data from all the haemopoietic cells, except CD14+ monocytes). Gene expression correlations were calculated and graphed using GraphPad Prism software. Correlation plots generated in R (v3.4.1) using the Corrplot package (v0.84); Pearson's R correlations calculated using stats::cor() and p-value matrix generated using corrplot::cor.mtest() (v0.84); heatmaps (i.e., Figure 6c) were generated using the ComplexHeatmap package (v1.18.0) (66). Standard student t test was used for all comparisons and Welch's correction applied when the variances between the tested groups were significantly different.

GEO2R(NCBI n.d.) was used to retrieve *Trib3* gene expression data from the microarray dataset GPL1261(36). This dataset comprises dormant or active LT-HSC, defined respectively as label retaining and non-label retaining CD34-Lin-Sca1+cKit+CD150+CD48- cells.

Conflict of Interest Statement
There is NO conflict of interest to disclose

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Figure legends

Figure 1. Summary BloodSpot datasets analysed. RMA: Robust Multiarray Averaging method. CvN: Cancer vs Normal method(47). Briefly, each cancer sample was mapped onto the gene expression-based map of the normal haemopoietic hierarchy, based on a two-steps PCA method, and gene expression fold changes were computed against the weighted average expression value of the 5 nearest normal samples. Comparison of gene expression values is not possible amongst the selected datasets. ImmGen: Immunological Genome project, H: human, M: murine, SNo: number of samples, N: normalisation. For further details refer to (28).

Figure 2. *TRIBBLES* expression in the human haemopoietic system. A) Log2 expression of *TRIB1*, *TRIB2* and *TRIB3* in normal human haemopoietic cells; the hierarchical expression trees show the relationship between the samples displayed. Expression range is denoted by the colour key, as indicated.

Expression in different population is based on curated microarray data: Human HSC cells are from GSE17054(29); Human GMP, MEP cells are from GSE19599(30); Human Monocytes cells are from GSE11864(31) and E-MEXP-1242(32). The hierarchical plots were generated using the BloodSpot platform(28). B) Data in A graphed as box with maximum and minimum whiskers. Midlines represent median values, boxes represent 25th-75th percentiles. The highest (red) and lowest (blue) median expression are indicated by the horizontal dashed lines. Significant gene expression variation of each population compared to all the other samples studied is indicated by *P<0.05, **P<0.01, ***P<0.001, with Student T test (black stars), or T test with Welch's correction for unequal variances (red stars).

Figure 3. *Tribbles* expression in the murine haemopoietic system. Log2 expression of *Trib1* (A), *Trib2* (B) and *Trib3* (C) in normal murine haemopoietic cells. Expression in different population is based on curated microarray data from GSE14833(34) and GSE6506(35) (Table 2). Midlines represent median values. The highest (red) and lowest (blue) median expression are indicated by the horizontal dashed lines. Significant gene expression variation of each population compared to all the other samples studied is indicated by *P<0.05, **P<0.01, ***P<0.001, with Student T test (black stars), or T test with Welch's correction for unequal variances (red stars). (D) TRIB3 expression levels in active and dormant LT-HSCs from microarray dataset GPL1261(36). *P<0.05, with Student T test.

Figure 4. *Tribbles* expression in the mouse haemopoietic system from the ImmGen dataset.A) hierarchical expression plots of *Trib1*, *Trib2* and *Trib3* in normal murine haemopoietic cells, the plots were generated using the BloodSpot database; colour code denotes expression, the range is indicated for each gene. B) Aligned dot plots representing data in A. Midlines represent median values. The highest (red) and lowest (blue) median expression are indicated by the horizontal dashed lines. Significant gene expression variation of each population compared to all the other samples studied is indicated by *P<0.05, **P<0.01, ***P<0.001, with Student T test (black stars), or T test with Welch's correction for unequal variances (red stars).

Figure 5. *TRIBBLES* expression pattern is non-redundant in human AML subtypes. *TRIBBLES* Log2 expression in normal haemopoietic cell (left from the black dashed line), AML subtypes, MDS and ALL (BloodPool datasets(28)) (right from the black dashed line). Data is graphed as box with maximum and minimum whiskers. Midlines represent median values, boxes represent 25th-75th percentiles. The highest (red) and lowest (blue) median expression are indicated by the horizontal dashed lines. Significant gene expression variation of each population compared to all the other samples studied is indicated by *P<0.05, **P<0.01, ***P<0.001, with Student T test (black stars), or T test with Welch's correction for unequal variances (red stars).

Figure 6. *TRIB1* and *TRIB2* expression have an inverse correlation in human AML. (a) Correlation between *TRIB1-TRIB2*, *TRIB2-TRIB3* and *TRIB1-TRIB3* expression in (i) normal haemopoietic cells, and in (ii) all AML, (iii) AML with MDS karyotype and (iv) ALL karyotype samples from the BloodPool dataset. Pearson's r correlation coefficient is represented as a colour gradient as shown in the bottom right of the figure. Associated P values are indicated (*P<0.05). (b) Correlations (Pearson's r) between *TRIB1* (red), *TRIB2* (blue) and *TRIB3* (green) and the *C/EBP* family members (*C/EBPα, C/EBPβ, C/EBPδ, C/EBPε*) are summarized as boxplots for the same clinical cohorts as shown in (a). Specific family members are labelled in supplementary Figure S5. (c) Heatmaps showing correlation (as measured by Pearson's r and coloured as in (a)) of TRIB1-3 with transcription factors known/predicted to influence expression of the TRIBBLES family. (i)-(iv) show these correlations for the same clinical cohorts shown in (a, b). The dendrogram to the left of each heatmap indicates similarity of correlation patterns of TRIB1-3. Columns represent the transcription factors of interest; these transcription factors are grouped into families using the colours shown on the top right hand side of the heatmaps.

Figure 7. *TRIBBLES* exhibit different expression FC in human AML subtypes. TRIBBLES fold change expression normalised to normal myeloid cells(47) across several AML subtypes, MDS and ALL (BloodPool vs normal dataset(28)). Data is graphed as box with maximum and minimum whiskers. Midlines represent median values, boxes represent 25th-75th percentiles. The highest (red) and lowest (blue) median expression are indicated by the horizontal dashed lines.

Significant gene expression variation of each population compared to all the other samples studied is indicated by *P<0.05, **P<0.01, ***P<0.001, with Student T test (black stars), or T test with Welch's correction for unequal variances (red stars). w denotes significant FC difference of median expression values compared to the baseline (0 FC), determined with Wilcoxon Signed Rank Test.

Figure 8. *TRIB1* and *TRIB2* expression is inversely correlated in human AML.Correlation between TRIBBLES expression in the BloodPool vs normal dataset, specifically in NK AML and samples with the most common chromosomal translocations. Pearson's r correlation coefficient is represented as a colour gradient as shown in the bottom right of the figure. Associated P values are indicated (*P<0.05).

Figure 1

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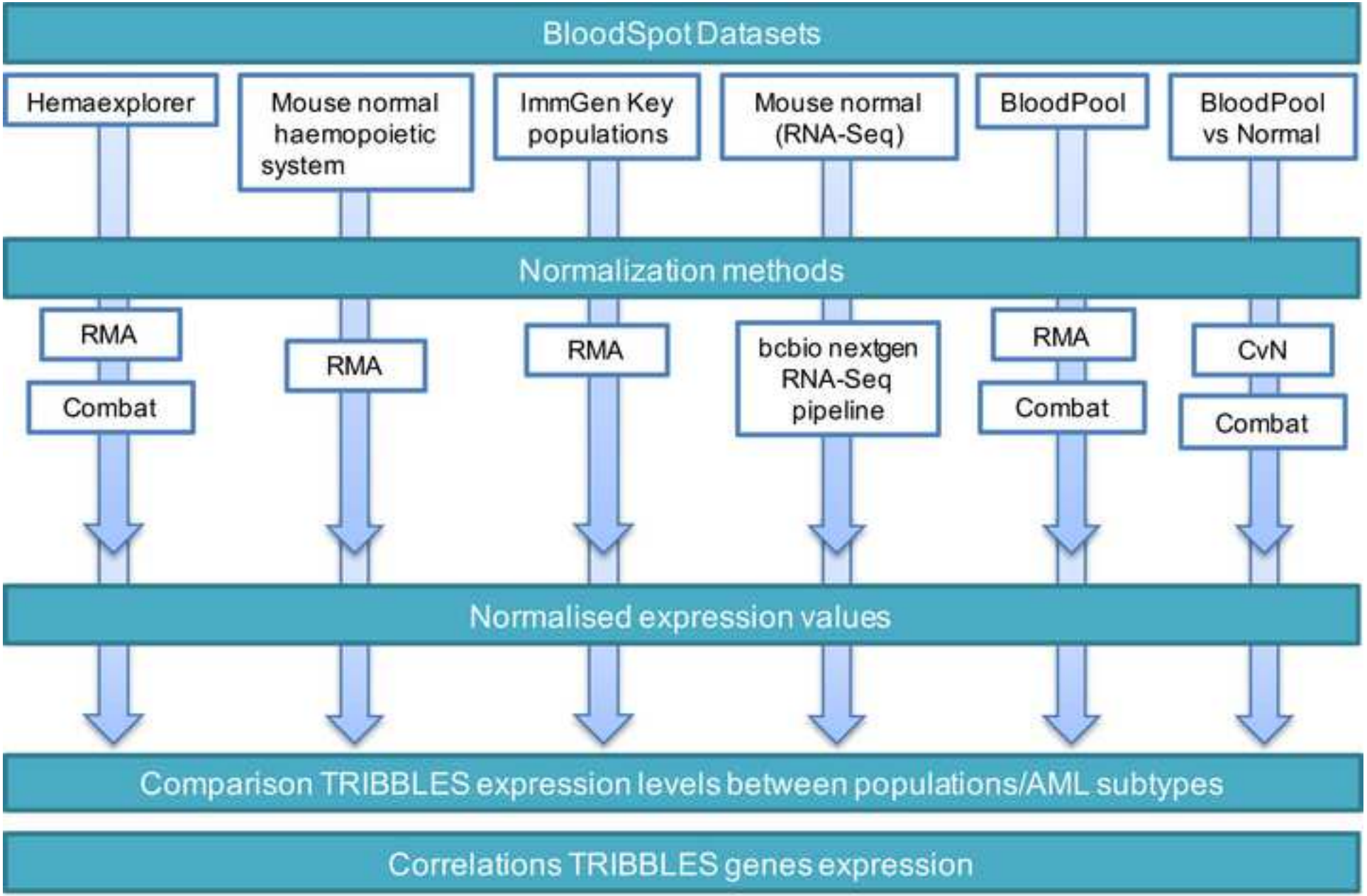

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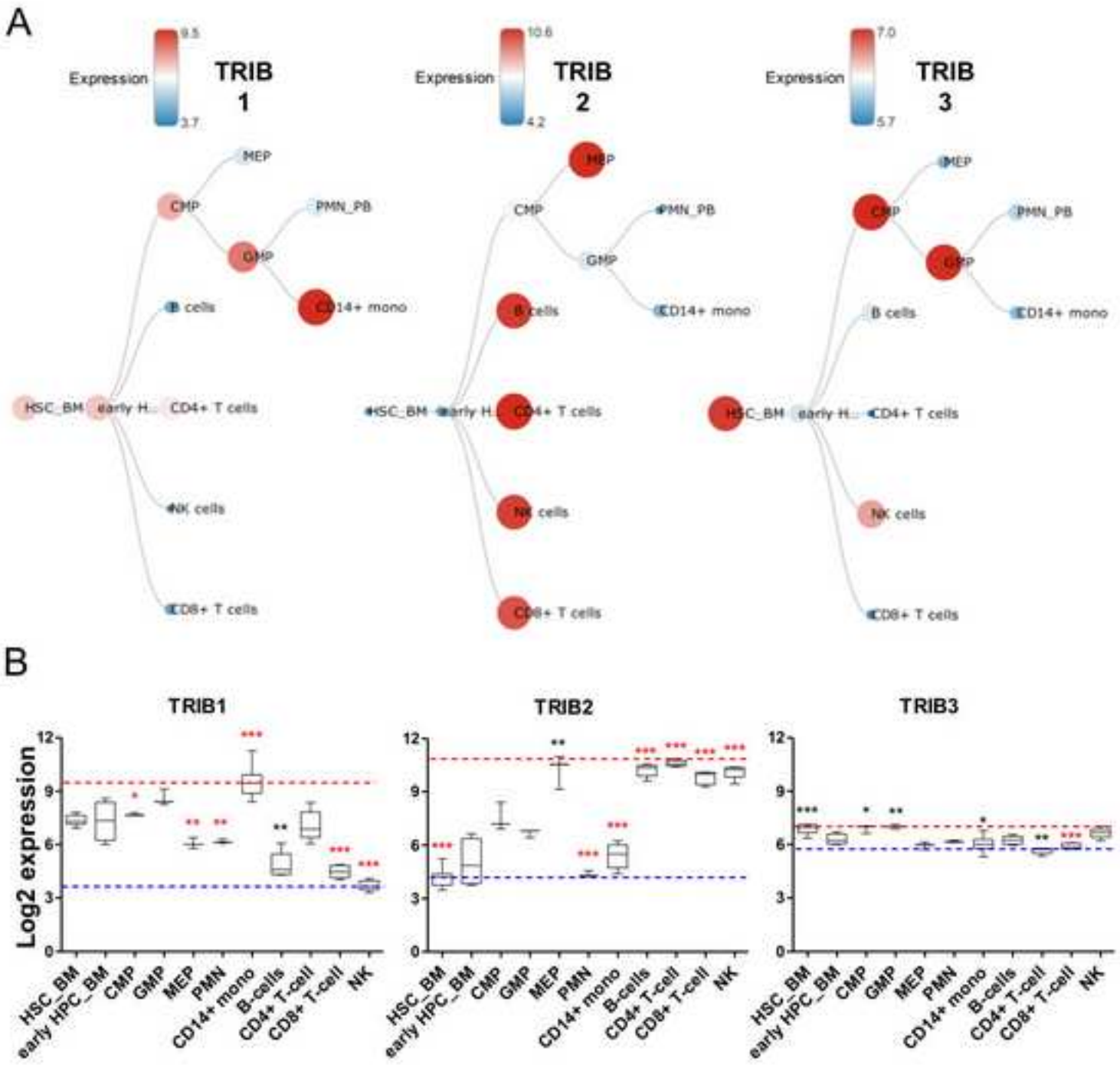

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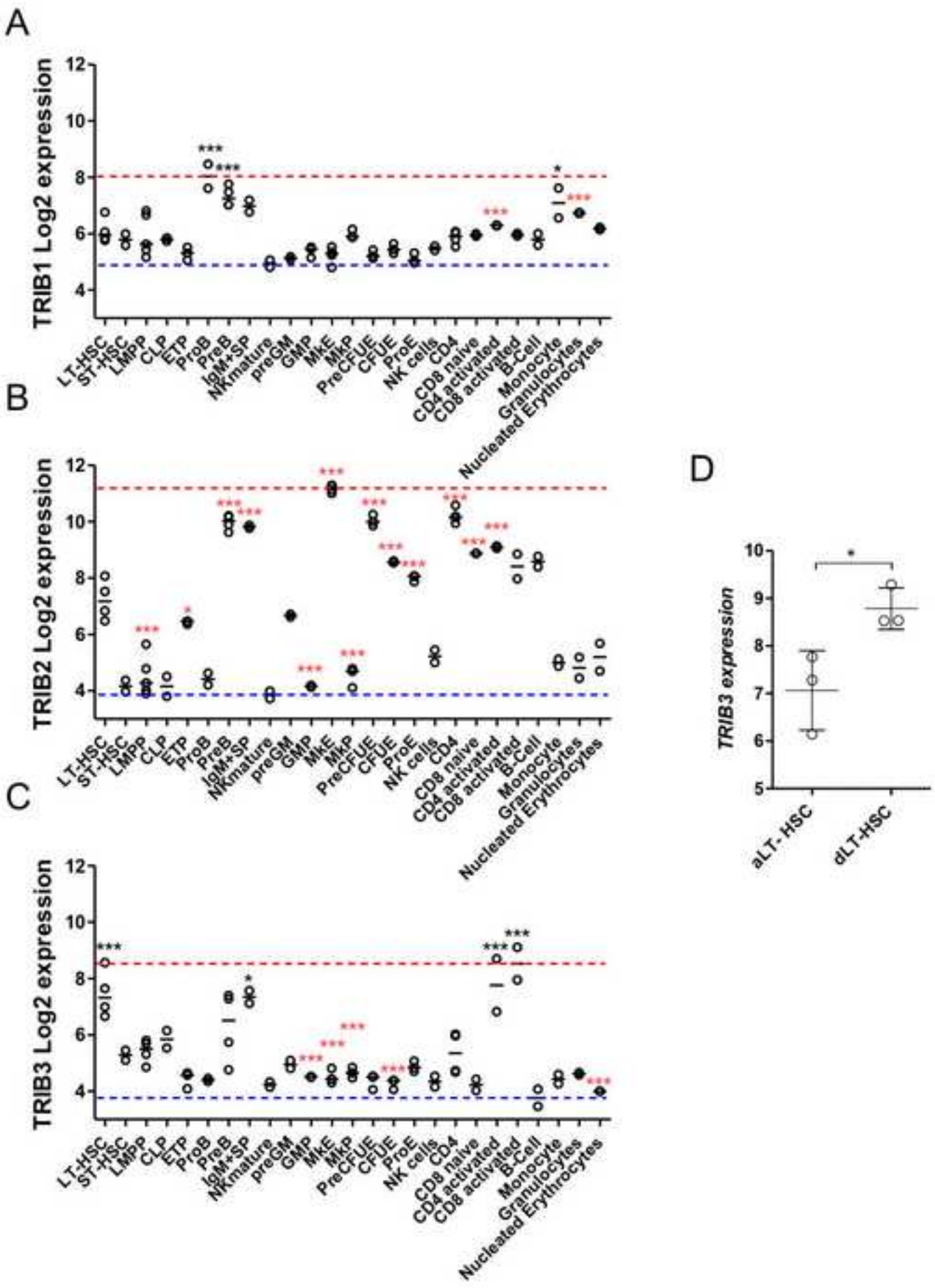

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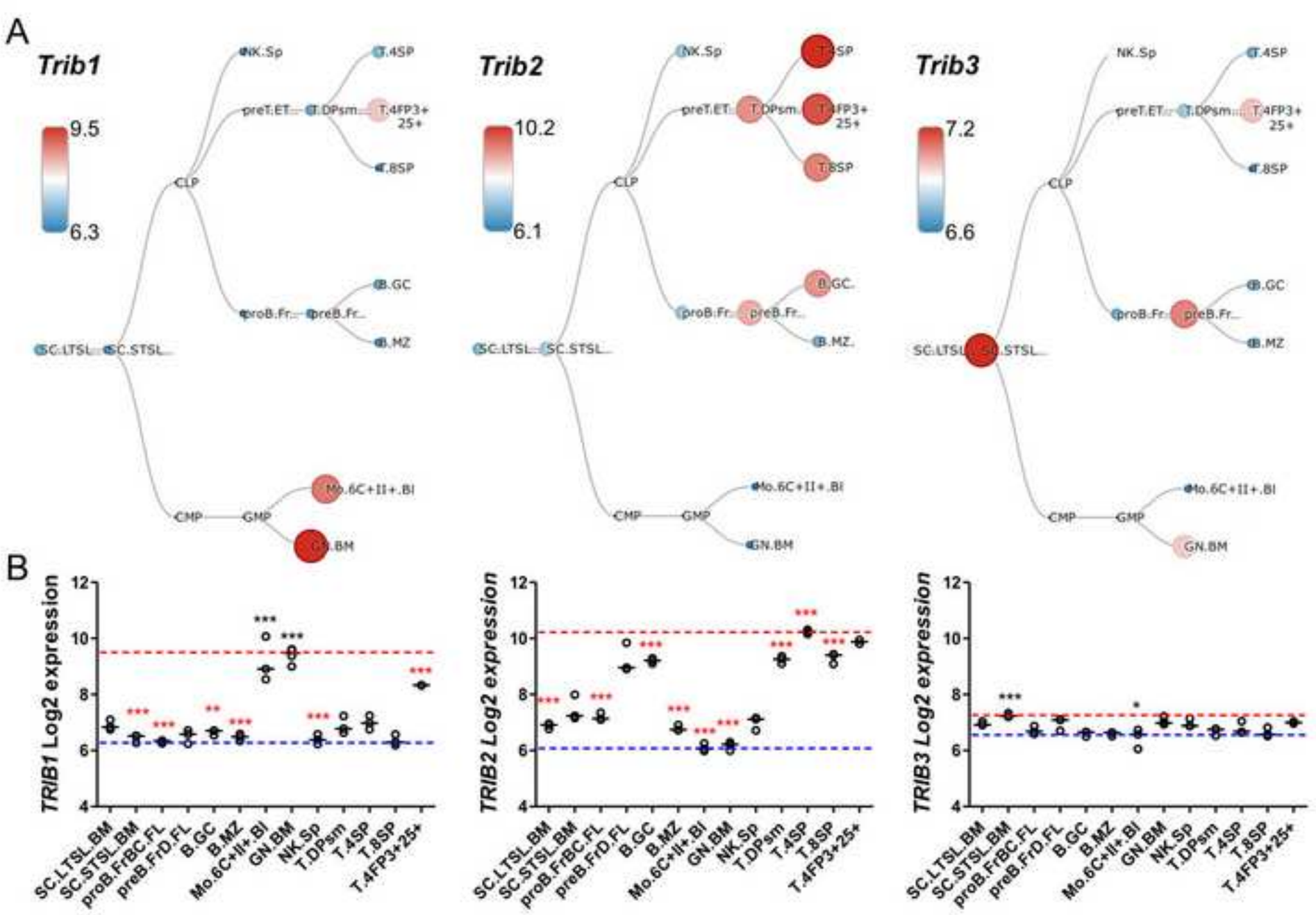

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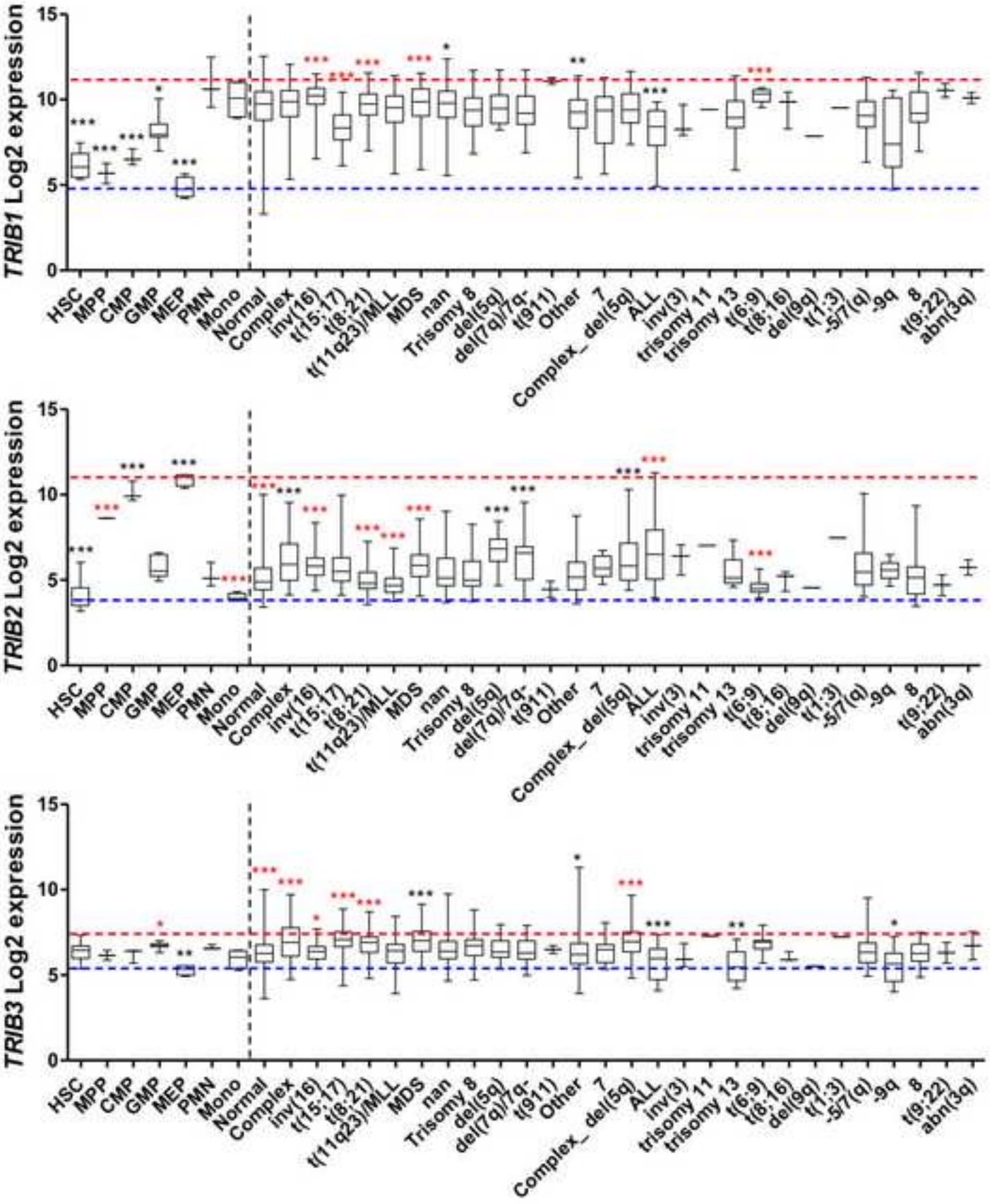

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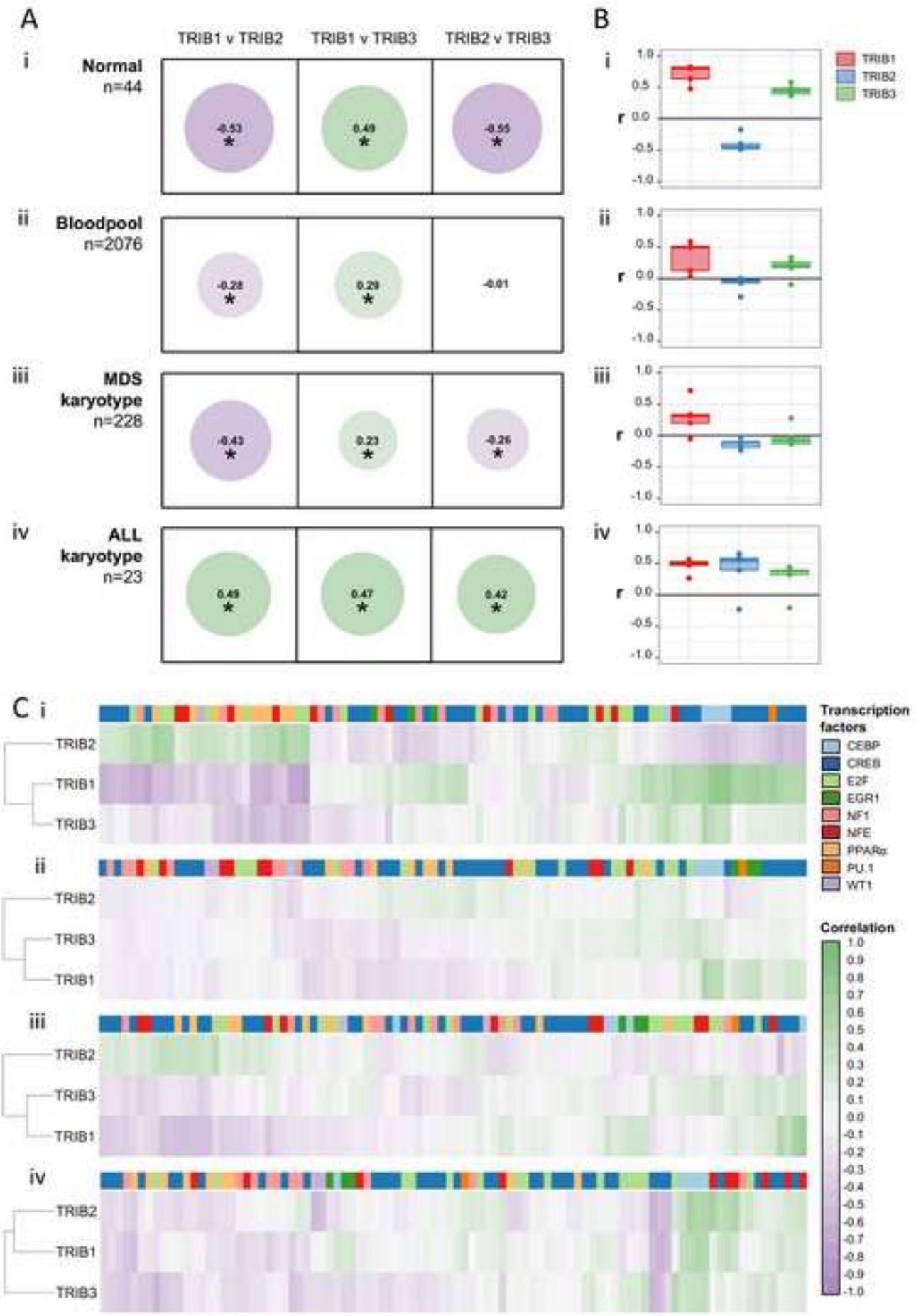

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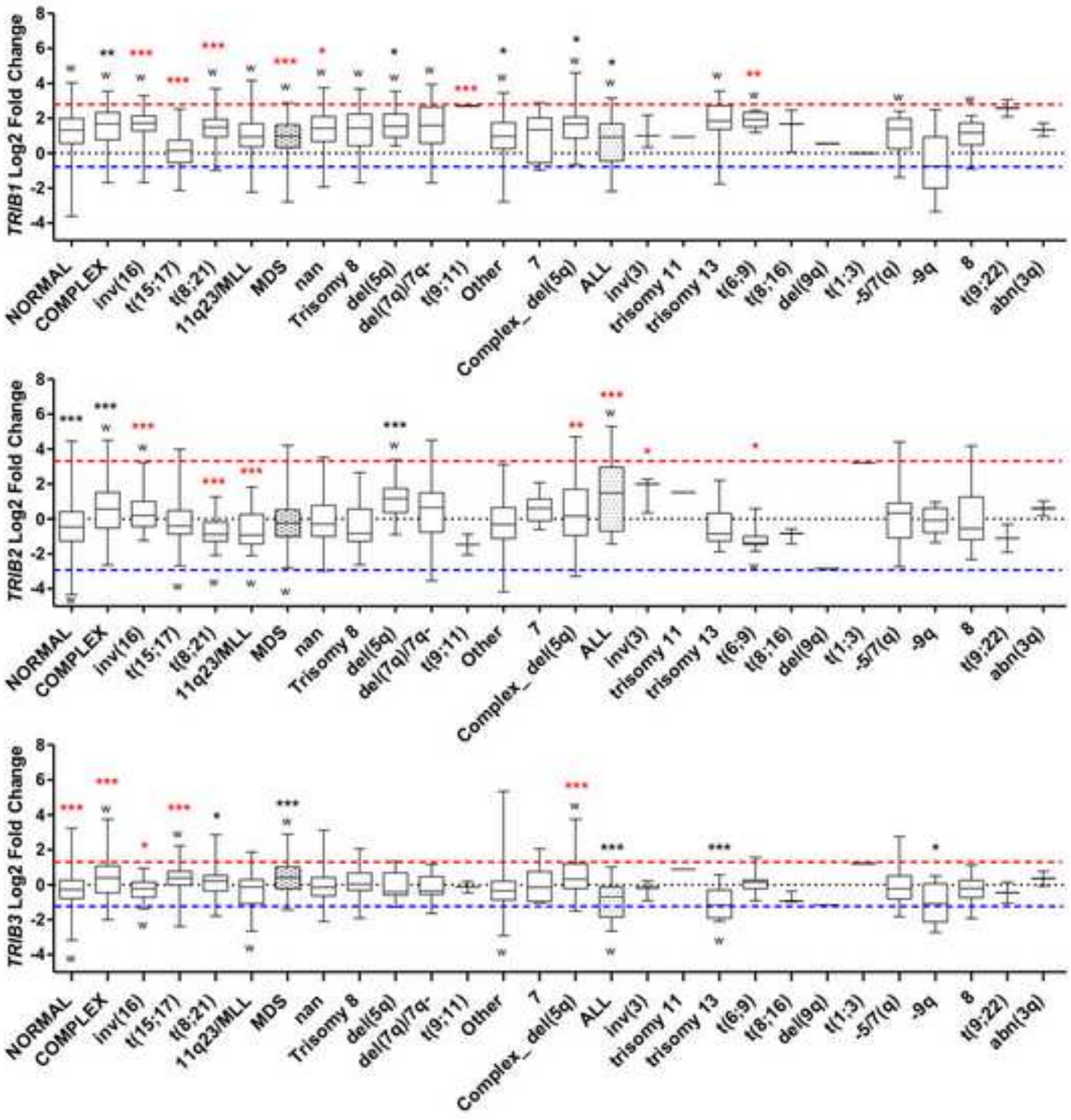

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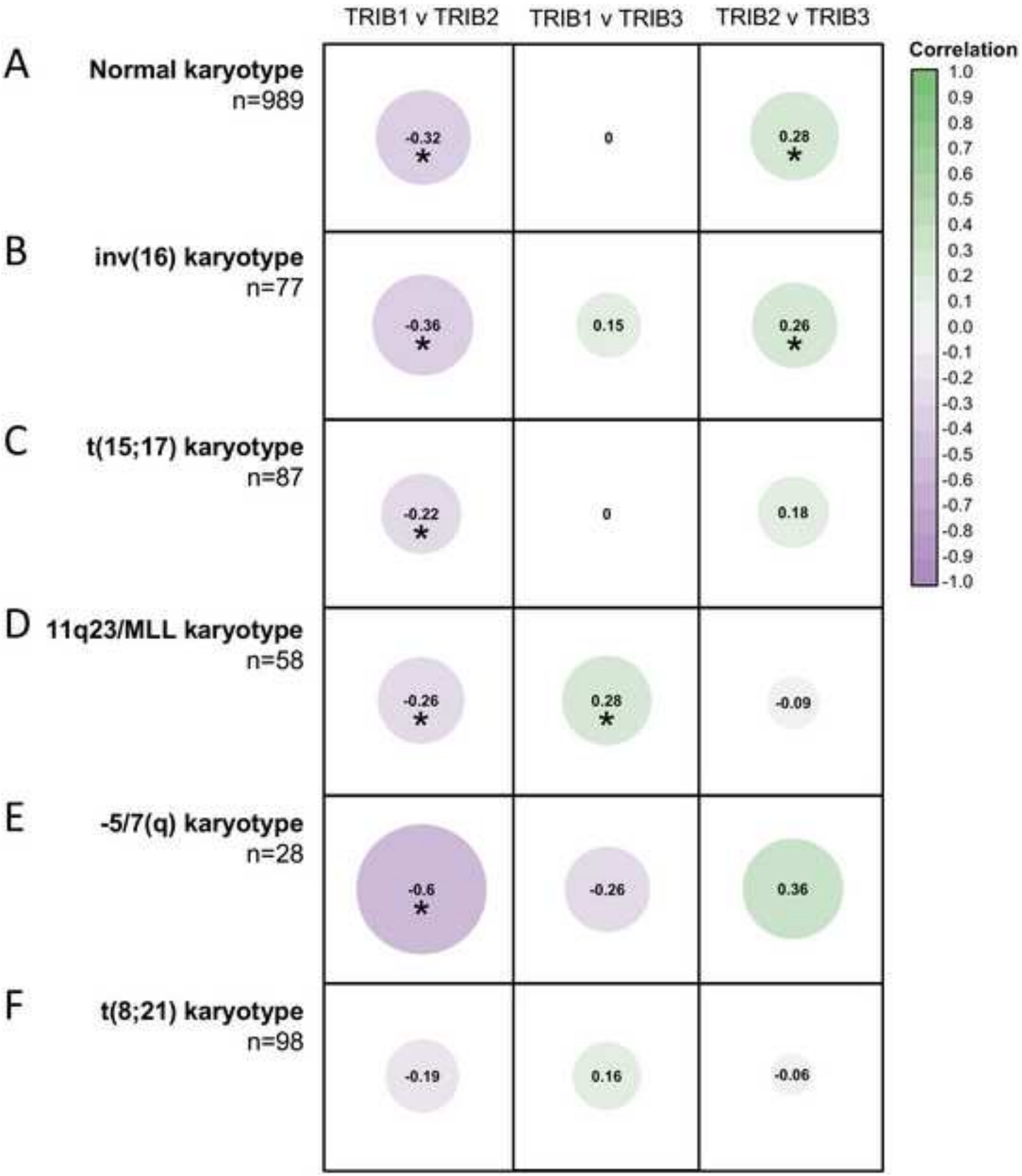

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